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BACTERIOLOGICAL STUDY OF FOUR CASES OF DIPHTHERIA TREATED WITH ANTITOXINE BY DR. LOUIS FISCHER AT THE MUNICIPAL HOSPITAL, PHILADELPHIA.

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While much has been written on laboratory technique in the study of the bacillus of diphtheria, yet the subject is of sufficient interest and importance to necessitate the publication of the methods employed in the study of these cases.

The bacillus of diphtheria grows best on blood-serum prepared after the method of Loeffler. The following was the mode of procedure in this laboratory study:

A wide-mouthed jar, with a ground-glass stopper, which has been thoroughly sterilized, is taken to the slaughter-house and filled with freshly-shed blood of the ox or sheep, young animals being preferable. The skin of the neck of the animal to be killed is shaved, washed with soap-water and a brush, then with alcohol or ether, or a 1:1000 solution of bichloride of mercury may be used if the skin is again washed thoroughly with boiled water. One of the great vessels of the neck, preferably an artery, is then opened, and the blood is allowed to spurt directly into the prepared receptacle. The edge of the jar is then cleansed and the jar is sealed by means of the ground-glass stopper, and set aside until the blood has firmly clotted. The jar is then removed to the laboratory, and placed in an ice-chest for twenty-four hours. It should be examined every few hours, and if the clot tends to adhere to the sides of the jar it should be loosened by means of a sterilized glass rod. There should be placed in the ice-chest at the same time the beef from which the nutrient broth is to be made. Take one pound of finely-chopped lean beef, to which is added one litre of water; this is allowed to stand for twelve hours. The meat and water are then placed in a cheese-cloth or towel and the fluid pressed out. The fluid thus obtained should be heated and again filtered through a towel, and the amount brought up to one litre by the addition of distilled water; to this is added 1 per cent. of peptone, 1 per cent. of glucose, and 0.5 per cent. of common salt. If the fluid has an acid reaction a few drops of saturated solution of bicarbonate of sodium may be added. The mixture is then boiled for half an hour and filtered through absorbent cotton into sterilized flasks; then sterilized for half an hour. While the serum is collecting test-tubes may be cleansed and sterilized. Two sizes of tubes are needed. A tube four inches long and two-thirds of an inch in diameter may be used for the serum, while a tube six inches long and

three-quarters of an inch in diameter is used for the cotton swab. The tubes are carefully washed and dried, then plugged with cotton so wrapped that its surface will be smooth, and when withdrawn from the test-tube will not spread out, but remain firm.

The tubes to be used for the swabs are prepared as follows: A stiff steel rod (an ordinary knitting-needle may be used) is passed through a plug of smaller diameter than the tube. The plug is wrapped so as to firmly hold the steel rod, which is passed through layers of cotton until the plug will tightly fit the calibre of the tube. The end of the rod which is placed in the tube is then wrapped with a small piece of cotton, which should be loose and fluffy at the end, but tightly adherent further up the rod. This arrangement can be effected by frilling the cotton and extending the fine fibres well up on the rod and wrapping firmly toward the end. The swab is now introduced into the tube, which is placed in an incubator and exposed to dry heat at a temperature of 150° C. for at least one hour. This should be repeated two hours before using the tube. The blood-serum is now siphoned off through a sterilized glass tube and mixed with the nutrient beef-broth in the proportion of 3 to 1. The serum is poured into the sterilized test-tubes by means of a sterilized pipet, which should be inserted to the bottom of the test-tube so as to avoid the formation of air-bubbles and to secure a perfectly even surface. Two c.c. of the serum are sufficient for each tube. The tubes are now placed in an incubator and kept at a temperature just below the boiling-point, care being taken to have them inclined in order to obtain a wide surface for the growth of the inoculated germs. Great care must be taken that the serum does not boil.

If kept for several hours at a temperature just below the boiling-point, and if the process is repeated two or three times after intervals of twelve hours, the serum will remain sterile. The serum thus prepared is opaque, yet firm. The tubes can be placed in glass jars, sealed by means of a clamp-top, and kept for months. The method given by Ball is much shorter and equally as good.

The swab for obtaining the membrane for inoculating the tubes is preferable to the inoculating needle, as the infected areas can be quickly passed over, and the secretions are readily gathered in the meshes of the cotton. Besides, the operator is not exposed so long in front of the patient.

The serum-tube may be brought to the bedside and inoculated at once after the swabbing of the throat, or the swab can be returned to the sterile tube and taken to the laboratory and the inoculation made there, and the serum-tube placed at once in the incubator.

A few words as to the method of obtaining the infective material from the throat and inoculating the tubes may not be amiss. With the patient in a strong light, the operator puts one hand on the patient's head, grasping the head firmly so as to control the movements of the patient should he cough, while with the other he holds the tube containing the serum. An assistant depresses the tongue, and with the other hand holds the tube containing the swab. The operator removes the swab and rubs it firmly but gently against any visible membrane, either

on the tonsils, pharynx, or uvula. It is advisable to make two tube inoculations, one from the well-formed membrane, the other from the ulcerated or oozing surface. The swab can now either be placed back in its tube, and the extending cotton singed and the whole be taken to the laboratory, or the serum-tube can be inoculated at once. After the swab has been used and the inoculation made, the cotton should be burned, while the steel rod can be again sterilized and used. Should no false membrane be visible the swab should be passed over the reddened mucous membrane. Should false membrane be present in the nose, inoculations should be made from it also. Inoculations should not be made for at least four hours after the application of disinfectants to the throat.

After the inoculation has been made, the serum-tubes are placed in the incubator and kept at a temperature of 37° C., the optimum temperature of the Klebs-Loeffler bacillus, for from ten to sixteen hours. After incubating the inoculated tube for ten or twelve hours the surface should be inspected, and as soon as the surface shows minute colonies, which spring up as almost pin-point elevations, a spread should be made. Great care must be taken not to spread the material too thickly on the cover-glass. This can be avoided by first placing a tiny drop of sterilized water on the cover-glass. The platinum needle is then introduced into the tube and swept over the colonies, and the bacteria adherent to the needle are washed off in the drop of water previously placed on the cover-glass, and evenly distributed over the entire surface.

The spread is then allowed to dry in the air, and before being placed in the stain is fixed by being passed quickly through the flame of an alcohol lamp. The spreads should then be stained with Loeffler's alkaline methylene-blue. The cover-glass is left in this stain without heating for from ten to fifteen minutes; it is then rinsed with distilled water, dried, and mounted in Canada balsam.

This brings us to the actual study of the individual inoculations. The cases are taken up separately. The report is mostly that of daily work in the laboratory, which was largely routine, being the examination of the inoculations made from day to day. As to proving in each case that the germ found was Klebs-Loeffler's germ, and virulent, inoculation of guinea-pigs would have been necessary. To do this with each daily inoculation would have been scarcely possible, at least not necessary, the standard germ being one which had been demonstrated beyond doubt to be Klebs-Loeffler's by producing the disease in animals. This was not necessary in two of the cases under observation, as they died of the disease. Tubes containing blood-serum prepared after the manner described by Loeffler were used in each inoculation.

In the four cases studied stains were made from each tube inoculated as soon as the growth appeared, also second stains were made after a

few hours which show more markedly the growth of other germs. The stain used was Loeffler's alkaline methylene-blue. The slides were all examined by Leitz $\frac{1}{12}$ oil immersion No. v. eye-piece.

CASE I.—F. N. (8668.) This case was examined by Dr. W. M. L. Coplin before injection with serum, and the Klebs-Loeffler bacillus was found in great numbers. The case was injected with serum, November 5th. Forty hours after injection a tube inoculation was made, which was incubated as described above, and after twelve hours a characteristic growth appeared. Stains from this showed many large Klebs-Loeffler bacilli, some showing the pole granules or refractile bodies, others showing small bacilli, rather pointed; a few streptococci were present, the Klebs-Loeffler presenting the same appearance. Stains made November 8th showed the Klebs-Loeffler bacillus more irregular and present in greater numbers, with increase in the number of streptococci, while the staphylococci remained about the same. Slides made November 10th showed an increase in the number of Klebs-Loeffler germs in the field as compared with other germs present on second stain; yet the first stain made from the tube showed nothing different from stains made November 8th. On November 10th the patient died. Total tube inoculations, six. Total stains made, thirty-six.

CASE II.—A. J. (8678.) Tube inoculations were made before injection with serum, November 5th. After incubating for sixteen hours, stains were made which showed the characteristic Klebs-Loeffler bacillus with few staphylococci. Later stains showed many cocci as well as the Klebs-Loeffler bacillus in large numbers, but somewhat irregular. Inoculations were made from this case as follows: November 7th, 8th, 10th, 11th, 12th, 13th, 15th, 16th, 17th, 18th, 19th, 21st, 23d, 24th, and 25th. It is not necessary to burden the reader with a description of each tube and slide; a statement of the general characteristics of the slides as the case progressed will suffice. On November 7th the slides showed very little difference from those made November 5th, except slight irregularity in the Klebs-Loeffler; on November 8th stains showed the beginning irregularity of the Klebs-Loeffler bacillus, many large and small, yet each showing some form of the germ. The cocci showed nothing different. No streptococci present.

November 10th, 11th, 12th, 13th were much the same, the Klebs-Loeffler bacilli being fewer in number, less irregular than in the earlier slides or in the other cases, and the cocci present in increased numbers, that is, by comparison. By some the irregular forms of the Klebs-Loeffler bacillus would possibly be described as another germ, but by closely watching the growth, and by isolation from the first growth appearing, the pure culture obtained showed the same characteristics. November 15th, 16th, 17th, and 18th showed practically the same, differing from the earlier tubes in that the Klebs-Loeffler was larger in size, showed the peculiar dumb-bell shape, some showing the refractile bodies, the germ being very irregular as to size and shape. A few bacilli somewhat resembling the Loeffler bacillus were present, but they did not present the characteristics. Stains from tubes inoculated November 19th, 21st, and 23d showed many cocci present, but fewer characteristic Klebs-Loeffler bacilli, also short rod-like bacilli which presented no characteristic appearance of being Klebs-Loeffler, either in appearance or by stain. The Klebs-Loeffler bacilli were markedly irregular and difficult to stain. Stains made November 24th and 25th

CASE A.

Fig. 1.

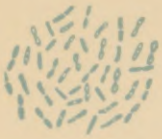


Fig. 2.

Fig. 3.

CASE B.

Fig. 1.

Fig. 2.

Fig. 3.

CASE C.

Fig. 1.

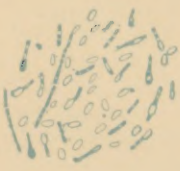
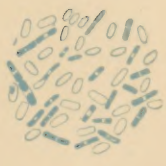


Fig. 2.

Fig. 3.



showed few Klebs-Loeffler bacilli, which were not so characteristic, and only an occasional one found present in the field. Many other non-characteristic bacilli were present as well as numerous cocci. Total number of tubes inoculated, seventeen. Total number of slides stained and examined, one hundred and ten.

Case III.—B. M. (8612.) Tube inoculations were made on November 5th, before the patient was injected with serum. After incubating for twelve hours spreads were made and stained. The field showed both strepto- and staphylococci, a short plump bacillus which tended to arrange itself in parallel rows, also the characteristic Klebs-Loeffler bacillus with its rounded ends, some swollen at one end, some at both, and some swollen in the middle, others presenting the refrac ile bodies. Stains made forty hours after the injection with serum showed on first and second stain the following: The number of cocci were increased as well as the number of short, thick, and deeply stained bacilli, while the Klebs-Loeffler bacilli were diminished in number and most markedly irregular as to size and shape; also more difficult to stain. Test-tube inoculations were made in this case as follows: November 7th, 8th, 10th, 11th, 12th, 13th, 15th, 16th, 17th, 18th, 19th, 21st, 23d, 24th. Stains made from tubes inoculated on the 8th and 10th showed considerable alteration in appearance from those made from tubes inoculated on the 7th, the slight alteration being on the 10th; the Klebs-Loeffler were slightly increased in number and more uniform; the cocci and short bacilli about the same. Stains made from inoculations made on the 11th, 12th, 13th, and 15th, showed nothing markedly different from those made on the 10th, except the presence of what I believe to be the *monilia candida* and a more irregular formation of the Klebs-Loeffler bacilli, yet they still presented their characteristic features. Stains made from the tubes inoculated on the 16th, 17th, 18th, and 19th showed gradual diminution in the Klebs-Loeffler bacilli. The germ showed irregular forms, many with swollen ends and many showing pole granules. The small thick bacilli increased in number from the 18th to the 21st, few streptococci, but many large and small staphylococci, many monilia candida present. Stains from tube inoculation made on the 21st, 23d, and 24th showed practically the same, except the gradual diminution of the Klebs-Loeffler bacilli with the irregular forms, yet there still could be found in every microscopic field some characteristic germs. Total tube inoculations made, sixteen. Total stains made, ninety-six.

Case IV.—J. H. (8689). This patient was injected with serum on November 9th. Stains from tube inoculation which was made before the injection showed as follows: From the first growth appearing the field was filled with characteristic Klebs-Loeffler bacilli, regular in size and shape, and few strepto- and staphylococci. Stains made a few hours later showed no diminution of the Klebs-Loeffler germ, but increase in the cocci, especially the streptococci, with a few non-characteristic bacilli. Stains made on the 10th showed increase in the non-characteristic bacilli, the cocci presented the same appearance, while the Klebs-Loeffler bacilli showed marked alteration in form, but no diminution as to number. Stains made on the 11th and 12th showed the same, while those made on the 13th and 15th showed an increase in the number of Klebs-Loeffler bacilli, although they were somewhat irregular. On the 15th the child died. Total tube inoculations, 6; total number of stains,

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36; total tube inoculations from four cases, 45; total stains made and examined, 278.

From each tube, after from six to ten days, second stains were made which in each case showed a diminution of the Klebs-Loeffler bacilli, with marked alteration, which alteration in form rendered the recognition of the germ almost impossible, with an increase in the non-characteristic bacilli and also of the cocci. This diminution and alteration in the Klebs-Loeffler bacilli was most marked in the tubes which contained no streptococci. Two cases were studied in which no serum was used. The tube inoculations were made at intervals to correspond with those made from the cases in which the serum was used. One case died after four inoculations, while the other improved, and the experiments were carried on at a length of time to correspond to the serum cases. These showed the characteristic germ present, yet from day to day they did not present such marked alteration in appearance as those from the serum cases, the Klebs-Loeffler bacilli remaining more uniform in appearance until after the disappearance of the membrane, when the non-characteristic germs were more numerous, cocci increased, and the Klebs-Loeffler diminished and more irregular. Stains made from the tubes of mixed growth of six days showed much the same as in the serum cases.

Conclusions.—The recognition of the bacillus of true diphtheria is somewhat difficult, owing to its resemblance to the germ described by Hofmann, yet I believe with others that with a careful examination of the bacterial growth in a blood-serum tube, which tube has been carefully inoculated according to the rules given above, then kept at first at a temperature slightly below the optimum, and yet not the minimum, for a few hours, then gradually brought up to the optimum and retained at that point for six or eight hours, then gradually lowered to about 32° C., the microscopic examination can be without doubt relied upon.

Roux and Yersin have shown that now and then bacilli from cases which clinically are diphtheria, and which show the Klebs-Loeffler germ, may possess little or no virulence; moreover, they also say, as quoted by Sternberg, "it is then possible by commencing with a virulent bacillus of diphtheria to obtain artificially a bacillus without virulence, quite similar to the attenuated bacilli which may be obtained from a benign diphtheritic angina, or even from the mouth of certain persons in good health. This microbe, obtained artificially, resembles completely the pseudo-diphtheritic bacillus; like it, it grows more abundantly at a low temperature; it renders bouillon more rapidly alkaline; it grows with difficulty in the absence of oxygen."

As to the significance of whether it is the Hofmann germ or Klebs-Loeffler much has been written, and authors differ widely in their views.

Personally I believe that the degree of resistance on the part of the patient has much to do with the variance in the germ. If in one case the Klebs-Loeffler germ fails to produce the disease when inoculated into animals, or the Hofmann's germ in one case causes the disease, it matters little in the care of the case whether it be the true or false germ—if there is a difference. Loeffler himself found the Klebs-Loeffler germ present in the case of a perfectly healthy child. Was the germ found virulent? Was it the germ of true or false diphtheria, or was it the resistance on the part of the patient, the healthy secretions of the mucous membranes, which lessened the virulence of the germ. In a certain number of cases which clinically are diphtheria the first inoculations may fail to show any Loeffler bacilli, yet a second inoculation made in a few hours will show many characteristic germs. It must be remembered that in each lot of test-tubes of culture media there are always some tubes in which no growth of any germ will develop. This is not the fault of the bacteriologist, but is due to the action of a material which forms within the tube, due to changes produced by excessive heating. What this material is has not been demonstrated, yet that it renders the culture media inert there is no question. Barring this one source of error, I think when the germ is not found in cases which are clinically diphtheria that the fault is on the part of the bacteriologist. It will be seen in the report of the cases in which the serum was used, that forty-eight hours after the injection of the serum the character of the germ was altered, yet it is not claimed that the antitoxine serum has any action directly on the germ; but by counteracting the poison in the system, produced by the product of the germ, the resistance on the part of the patient manifested at the nidus of infection indirectly affected the germ's nutrition, thereby altering its character. This is true, providing the previous products have not caused pathological alteration in tissue structure; in such cases it is not possible for the serum to effect such alterations. At one time it was thought by some observers that the size of the germ had to do with its virulence. It is now conceded by most observers that the size and length of the germ have nothing to do with its virulence. Certainly in these cases studied, which clinically were most severe in character, no definite conclusion could be made from the appearance of the germs. They were irregular in size in each case. Strepto- and staphylococci live long in the throats of healthy individuals without causing any disturbance so long as the mucous membranes are healthy; but under certain conditions, as when the mucous membrane's resistance has been lessened by exposure to cold or other deleterious influences, or by the poison of scarlet fever or other infectious diseases, the streptococci alone or associated with other germs attack the mucous membranes and cause inflammation; it has been shown that when there are cases of

scarlet fever in the same institution, the Klebs-Loeffler bacilli may be found in the pseudo-membrane forming in such cases.

It is a well-known fact that environments alter the characteristic features of all germs; that in the description of a germ, temperature, light, culture medium, and absence or presence of other bacteria must be taken into consideration. Also that laboratory germs which depend on artificial nutrition differ somewhat from those found in the body; this is especially true of the bacillus of diphtheria, which is demonstrated by the difference in description given by various authors; the alteration of the Klebs-Loeffler bacillus, as due to the age and conditions under which the germ was found and grown, may be shown in the following plates, the legend explaining the growth. The germs were subjected as nearly as possible to the same conditions, all being grown on Loeffler's blood-serum, except Case C, Fig. 1.

Case A, Fig. 1. Tube inoculated before serum injection. The diphtheria bacilli are seen as irregular staves, dumb-bell shape, with refractile bodies at the poles, and unevenly stained staves, the poles staining intensely while the central portion is pale. (Eye-piece iv., Beck objective $\frac{1}{12}$ ol. im. Leitz.)

Case A, Fig. 2. Tube inoculated forty hours after serum injection. The diphtheria bacilli have lost some of their irregular form; though still markedly irregular, some are now seen as short staves which stain intensely at the poles.

Case A, Fig. 3. Tube inoculated five days after tube 1. The bacilli are larger and more irregular than in tube 2, though they show otherwise the same characteristics.

Case B, Fig. 1. Tube inoculated upon the admission of patient. No serum was used. The diphtheria bacilli are slightly irregular in form, though the larger number are small staves staining intensely at the poles.

Case B, Fig. 2. Tube inoculated forty hours after admission. The diphtheria bacilli are smaller and more regular in form than the preceding. They stain intensely at the poles.

Case B, Fig. 3. Tube inoculated five days after admission. The diphtheria bacilli are slightly larger than in tube 2, otherwise they appear the same.

Case C, Fig. 1. From agar-agar tube of Klebs-Loeffler bacillus six months old. The bacilli are shown as irregular staves, staining unevenly. The staining is quite intense at the poles. In some few of the bacilli one end is greatly enlarged. There are present, also, a large number of ovoidal bodies which stain only at the periphery.

Case C, Fig. 2. Tube 2, inoculated from tube 1, growth forty-eight hours old. Irregular staves, staining for the most part very unevenly.

The bacilli seem to tend to the formation of short chains. Few ovoidal bodies are present.

Case C, Fig. 3. Tube 3, inoculated five days later from tube 2. The bacilli closely resemble those in tube 1, though the ovoidal bodies are not so numerous.

It will be seen that the most marked change occurred in the bacilli from the serum case and from the old culture. In many slides presenting the irregular involution forms it would be difficult to distinguish many of the germs from the germ described by Hofmann.

If, when the germ is grown on different nutrient media its form is so markedly altered, certainly we must grant that in different individuals the germ would be placed under equally as great variations in nutrition, besides the resistance on the part of the patient.

From the drawings made from the stains which were made from growths subjected as nearly as possible to the same conditions, it is quite clear why it is so difficult to distinguish the *true* from the pseudo-diphtheritic bacillus. It is impossible to differentiate microscopically.

That the Klebs-Loeffler bacillus exists in the throats of healthy indi viduals who have been exposed to diphtheria has been demonstrated by many observers. It may be interesting to note that in tube inoculations made from the throats of individuals exposed to diphtheria while examining the cases in the hospital ward, the characteristic Klebs-Loeffler bacilli were found; the virulence was not tested.

All bacilli found in cases which are clinically diphtheria, which bacilli are identical with the Klebs-Loeffler bacillus, should be classed with the diphtheria bacillus, even if their virulence has not been tested on guinea-pigs, or even if tested and little or no virulence is shown.

In three cases streptococci were found. Two of these cases proved fatal; one in which a few were found recovered. One thing noticeable was the absence of other germs besides those mentioned, which was practically the case when the Klebs-Loeffler bacilli were present in large numbers.

I am indebted to my laboratory assistants for their help in these investigations.

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